consumption and lactate production and the presence of mitochondrial dysregulation in PRT-treated PLTs were reported.[7,8] There have been various reports on the alteration of PLT functions caused by PRT treatment. The PLT aggregation in response to collagen and thrombin receptor–activating peptide (TRAP) was reported to be lesser in PRT-treated PLTs than in untreated control PLTs.[9] On the other hand, the adhesion of PRT-treated PLTs to assay plates was shown to be greater than that of control PLTs by the results of thrombus formation assay using Impact-R (DiaMed, Cressier sur Morat, Switzerland), which reflects the PLT ability for adhesion and aggregation associated with a shear stress caused by blood flow.[10] Moreover, using a perfusion model with rabbit aorta segments, the thrombus formation after storage was better maintained in PRT-treated PLTs than in control PLTs.[11] It seems that results in regard to the effects of PRT treatment on PLT functions vary depending on the assay methods used. In single-blind crossover studies, in vivo recovery and survival of PRT-treated PCs were reduced compared with those of untreated control units, as evidenced by reduced corrected count increment.[12,13] However, the results of a recent pilot study of PLT function in PRT-treated PLTs isolated from the circulation of patients after transfusion have suggested that these circulating cells may elicit hemostatic responses comparable to those in untreated PLTs.[14] To develop strategies that minimize the negative effects of PRT, it is necessary to determine the mechanisms by which PLTs are damaged.

In this study, we prepared columns filled with collagen-coated beads and performed a thrombus formation assay to clarify the effects of PRT treatment on PLT functions. In our collagen column method, thrombus formation on the solid-phase collagen similar to that observed in the damaged vascular subendothelium was assayed at a constant shear rate. This method better reflects physiologic conditions than PLT aggregometry, in which PLTs are simply stirred in the presence of agonists. Moreover, the collagen column method utilizes PLT-rich plasma (PRP), which is different from other methods using a flow chamber or Impact-R that require reconstituted blood or whole blood samples. Using the collagen column method, we determined the effects of PRT treatment on PLT thrombus formation and examined the mechanism underlying the effects.

MATERIALS AND METHODS

Materials

Common chemicals were purchased from Sigma-Aldrich (St Louis, MO) or Wako Pure Chemicals (Osaka, Japan). Copolymer plastic beads (165 μm in diameter on average) coated with porcine Type I collagen were purchased from ISK (Tokyo, Japan). A monoclonal Alexa Fluor 488–conjugated CD41 antibody (Clone MEM-06) was purchased from EXBIO (Prague, Czech Republic). A monoclonal fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody was purchased from BD Biosciences (San Jose, CA). A monoclonal phycoerythrin (PE)-cyanin 5.1 (PC5)–conjugated anti-CD41 antibody (Clone P2) was purchased from Beckman Coulter (Fullerton, CA). Polyclonal FITC-conjugated anti-human fibrinogen antibodies were purchased from Binding Site (PF056, Birmingham, UK) and Agrisera (IMS09-038-335, Vannas, Sweden).

Preparation of PCs

Informed consent was obtained from all healthy volunteers before apheresis (AP) donation. Leukoreduced AP-PCs (volume of 230 ± 16 mL and yield of $3.4 \times 10^{11} \pm 0.8 \times 10^{11}$ PLTs/bag) were collected using automated blood collection systems (Trima Accel, Terumo BCT; or CCS, Haemonetics, Braintree, MA).

PRT treatment

The Mirasol PRT treatment (n = 32, blood group composition: A, 12; B, 3; O, 17) was performed on Day 1 postcollection as described elsewhere.[6,8,15] Briefly, after the addition of 35 mL of riboflavin solution to an AP-PC bag at a final concentration of 50 μmol/L, the bag was exposed to UV light at a dose of 6.24 J/mL (265-370 nm) and allowed to stand for 30 minutes before placement on a flatbed agitator running at 55 agitations per minute. To the untreated control bag (n = 17, blood group composition: A, 4; B, 1; O, 12), 35 mL of a 0.9% saline solution was added. All PCs were kept at 20 to 24°C on the flatbed agitator for 5 days. All PLT samples were taken from bags, under sterile conditions, at 2 hours after PRT treatment and after 3 and 5 days of storage.

Thrombus formation assay and fluorescence microscopy

Collagen columns were prepared by filling silicon tubes (4-mm outer diameter, 2-mm inner diameter, and 50-mm length) with copolymer plastic beads coated with porcine Type I collagen using dry air (9 mL/min, 10 min) from an air compressor. Thus-prepared columns were set in an incubator at 37°C. PRP (30×10^{10} PLTs/L) prepared from PCs was incubated at 37°C for 10 minutes, and 0.5 mL of PRP was then passed through the columns at a shear rate of 750 per second by aspirating with a syringe pump (Fig. 1). This shear rate was calculated on the basis of previous reports.[16,17]

Retention rates obtained from PLT counts before and after the passage of PRP through the columns were used as indices of thrombus formation. All of the PRP samples before and after passage through the columns were

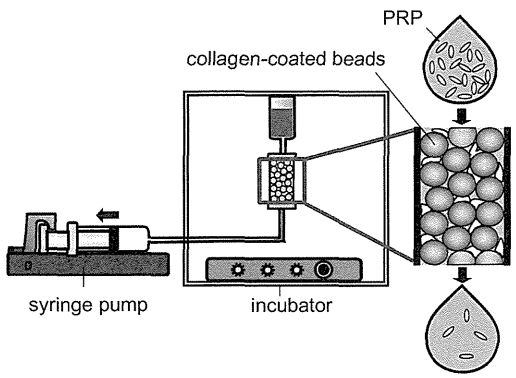

Fig. 1. Schematic diagram of collagen column system used for thrombus formation assay.

collected into plastic tubes containing ethylenediaminetetraacetic acid (5 mmol/L), and PLT count was determined using an automatic blood cell counter (XS-800i, Sysmex, Kobe, Japan). PLT retention rate (%) was calculated as follows: [(total PLT count before passing through the column) – (total PLT count after passing through the column)]/(total PLT count before passing through the column) × 100.

In one experiment, beads were taken from the columns through which PRP was passed and fixed with 1% paraformaldehyde phosphate-buffered saline (PBS) for 1 hour. The beads were then fluorescently stained with an Alexa Fluor 488–conjugated anti-CD41. The fluorescent images of PLT thrombus formed on the beads were observed by fluorescence microscopy (IX-71, Olympus, Tokyo, Japan) at 40× magnification.

Flow cytometry

Total αIIbβ3 level was measured on the basis of the level of binding of the anti-CD41 (P2) to the αIIbβ3 complex. The state of αIIbβ3 activation was evaluated on the basis of PAC-1 binding or fibrinogen binding. PLT samples were incubated with an FITC-conjugated PAC-1 antibody or FITC-conjugated polyclonal anti-human fibrinogen antibodies and a PE-cyanin 5.1–conjugated anti-CD41 for 20 minutes at room temperature. Isotype control antibodies were also included as controls. The samples were fixed with 1% paraformaldehyde PBS and analyzed by flow cytometry using the accompanying software (Cytomics FC500 and CXP software Version 2, respectively, Beckman Coulter, Miami, FL). Fluorescence data from 10,000 PLT events were collected in the logarithmic mode.

To examine the maximum binding of fibrinogen to PLTs, PLT samples, either PRT treated or untreated, were stimulated with 100 μmol/L TRAP for 5 minutes and stained with an FITC-conjugated anti-fibrinogen antibody and then subjected to flow cytometry analysis.

Mn^{2+} treatment

To clarify the effect of αIIbβ3 activation on thrombus formation, in some experiments, PRP samples were incubated with 1 mmol/L $MnCl_2$ as the activating agent at 37°C for 5 minutes before the thrombus formation assay and flow cytometry. As previously shown by others, this concentration of Mn^{2+} can fully activate αIIbβ3.[18,19]

Statistical analysis

Results are presented as mean ± SD. The t test was conducted with p values of not more than 0.05 indicating a significant difference. Correlations were determined using Spearman's rank correlation method. A correlation coefficient of more than 0.75 between methods was considered to indicate a good to excellent relationship. A correlation coefficient from 0 to 0.25 indicated a poor or no relationship.[20]

RESULTS

Effects of PRT treatment on PLT thrombus formation

We measured retention rate as an index of thrombus formation by the collagen column method. The retention rate of the PRT-treated PLTs on the day of treatment (Day 1) was 97.3 ± 6.1%, which was significantly higher than that of the control PLTs, 39.5 ± 21.7%, by more than twofold (Fig. 2A). Riboflavin alone without UV radiation did not increase the retention rate (46.1 ± 23.3%, n = 7, data not shown). We also observed the fluorescent images of PLT thrombi formed on the collagen-coated beads taken from the columns through which PRP passed. There were more PLTs and larger thrombi that covered multiple beads through which the Day 1 PRT-treated PLTs passed than on the beads through which the control PLTs passed (Fig. 2B). The retention rate of the PRT-treated PLTs was highest on Day 1 and decreased with storage period. The retention rate of the PRT-treated PLTs was significantly higher than that of the control PLTs throughout the storage period (Fig. 2A). These findings showed that PRT treatment enhanced the thrombus formation of the PLTs on collagen and that the thrombus formation was well maintained during the storage period.

Effects of PRT treatment on αIIbβ3

αIIbβ3 molecules on the PLT surface play a crucial role in thrombus formation. We examined the effects of PRT treatment on αIIbβ3 using the P2 antibody, which

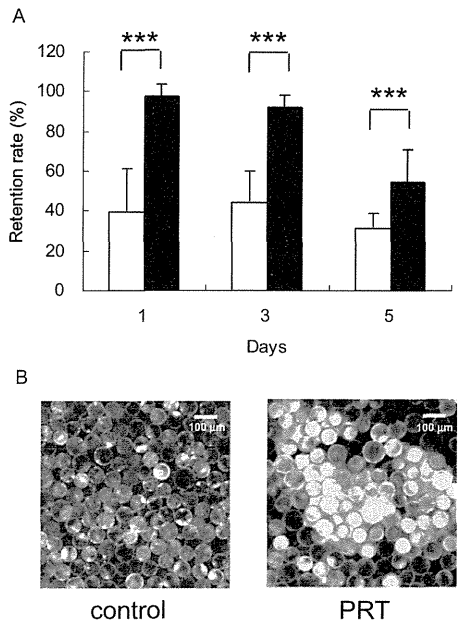

Fig. 2. Effects of PRT treatment on PLT thrombus formation. (A) Thrombus formation by collagen column method: control (□), PRT treated (■). (B) Typical fluorescent image of thrombus on collagen-coated beads through which Day 1 PLTs passed: (left) control; (right) PRT treated. Thrombus formation was determined on the basis of retention rate (%) presented as mean ± SD. n = 17 (□); n = 32 (■). ***p < 0.001.

recognizes the αIIbβ3 complex; the PAC-1 antibody, which recognizes conformational changes caused by αIIbβ3 activation; and the anti-fibrinogen antibody. There was no significant difference in the total αIIbβ3 level between the PRT-treated PLTs and the control PLTs on Day 1 (Fig. 3A). From the third day of storage, the total αIIbβ3 level on the PRT-treated PLTs was significantly higher than that on the control PLTs. The PAC-1 binding on the PRT-treated PLTs was significantly higher than that on the control PLTs (85.3 ± 9.4% vs. 10.7 ± 7.1%) on Day 1 (Fig. 3B) and decreased with storage time while maintaining a higher level than that on the control PLTs throughout the storage period. Using PF056, the level of fibrinogen binding on the PRT-treated PLTs (5.26 ± 1.34) was significantly higher than that on the control PLTs (4.08 ± 0.61) on Day 1 (Fig. 3C). However, it gradually increased with storage time compared with the control, which is in marked contrast to PAC-1 binding which shows a decreasing trend with storage time. The increasing trend of fibrinogen binding was confirmed with the other polyclonal antibodies specific for fibrinogen (data not shown). When the Day 1 PRT-treated PLTs were stimulated by TRAP, fibrinogen binding level markedly increased by 10.6- and 7.9-fold in terms of mean fluorescence intensity for control and PRT-treated PLTs, respectively (n = 4, data not shown), indicating that the fibrinogen binding is only partial although it increases with storage time. These findings show that PRT treatment immediately activated αIIbβ3 and sustained αIIbβ3 activation during the storage period; however, the sustained αIIbβ3 activation did not correspond to the increasing trend of total αIIbβ3 level and fibrinogen binding level.

Correlation between thrombus formation and activation of αIIbβ3

We compared the retention rate measured by the collagen column method with the activation marker measured by flow cytometry for all the PRT-treated and control PLT samples to determine the correlation between thrombus formation and αIIbβ3 activation (n = 49; Table 1). There were no correlations between retention rate and total αIIbβ3 level (P2 binding) or fibrinogen binding level (Spearman $r < 0.25$). However, there was a significant correlation between retention rate and αIIbβ3 activation (PAC-1 binding; Spearman $r = 0.840$, $p < 0.001$). These findings suggest that αIIbβ3 activation affected the thrombus formation as verified by the collagen column method, whereas the thrombus formation did not correlate with the total αIIbβ3 level or fibrinogen binding level.

Effects of Mn^{2+} on αIIbβ3 and thrombus formation

We measured the retention rate and PAC-1 binding level in the Day 5 PRT-treated PLTs and control PLTs after incubation with 1 mmol/L $MnCl_2$ at 37°C for 5 minutes to determine whether thrombus formation is restored by the reactivation of αIIbβ3. Mn^{2+} directly activates αIIbβ3 extracellularly without being mediated by signals from inside to outside the PLTs. The PAC-1 binding level in the PRT-treated PLTs significantly increased from 36.2 ± 19.2% to 95.6 ± 2.8% whereas that in the control PLTs also increased from 8.5 ± 6.5% to 66.9 ± 22.7% (Fig. 4A). The retention rate in the PRT-treated PLTs significantly increased from 54.3 ± 16.5% to 88.5 ± 15.6% whereas that in the control PLTs increased from 31.3 ± 7.7% to 82.5 ± 12.3% (Fig. 4B). As is the case for the Day 1 PRT-treated PLTs, there were many PLTs and large thrombi adhering to multiple beads through which the PRT-treated PLTs and control PLTs passed after the addition of Mn^{2+} (Fig. 4C). These findings suggest that thrombus formation on collagen was restored owing to the reactivation of αIIbβ3 in the PRT-treated PLTs after the addition of Mn^{2+}.

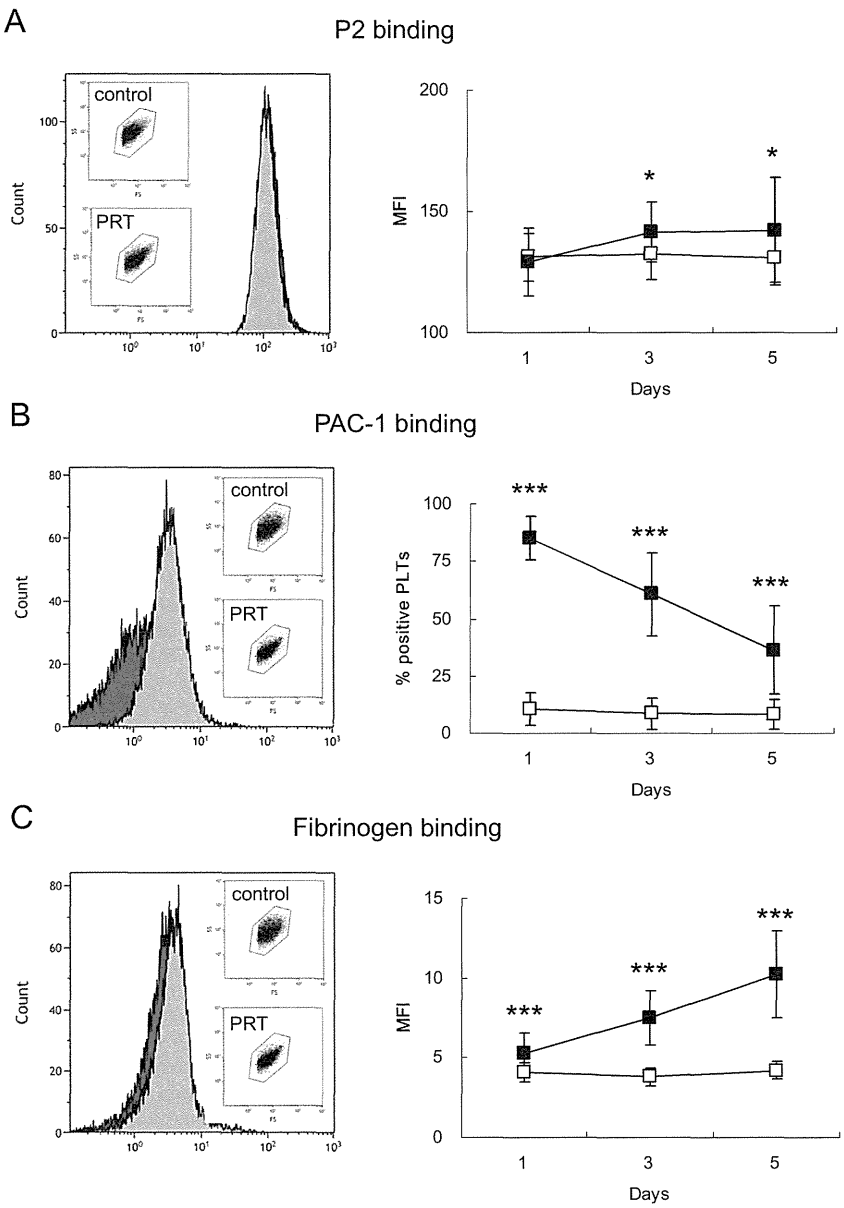

Fig. 3. Effects of PRT treatment on αIIbβ3 level and fibrinogen binding as determined by flow cytometry. (A) Total αIIbβ3 level represented as CD41 positivity; (B) activated αIIbβ3 represented as PAC-1 binding; (C) fibrinogen binding. Figure 3C shows representative results obtained using the fibrinogen-specific antibody PF056, but the same results were obtained using the other antibodies specific for fibrinogen (IMS09-038-355). Representative histograms (left column) indicate P2, PAC-1, and fibrinogen binding on the Day 1 control (▩) and the Day 1 PRT-treated (□) PLTs. Boxes in each graph (right column) indicate the values for the control (□) and the PRT-treated (■) PLTs presented as mean ± SD. (Insets in histograms) Dot plots of forward scatter versus side scatter of a typical sample, with the gate set to exclude small vesicles and large aggregates. n = 17 (□); n = 32 (■). MFI = mean fluorescence intensity. *p < 0.05; ***p < 0.001.

TABLE 1. Correlation between retention rates and the flow cytometer variables

Variable	*r*	p value
P2 binding (MFI)	0.067	0.420
PAC-1 binding (% positive PLTs)	0.840	<0.001
Fibrinogen binding (MFI)	0.234	0.004

MFI = mean fluorescence intensity; *r* = Spearman's rank correlation coefficient.

DISCUSSION

With the aim of clarifying the effects of PRT treatment on thrombus formation in PLTs, we performed a thrombus formation assay using the collagen column method. This method was designed to assess PLT function in terms of thrombus formation under nearly physiologic conditions rather than turbidometric aggregometry, in which PLTs are stimulated by agonists under stirring condition. The collagen column method had also been used in a clinical setting for the evaluation of dose dependency of anti-PLT agent.[17] We found that thrombus formation in the PRT-treated PLTs was enhanced under flow in the collagen column. The enhanced thrombus formation was well sustained during the storage period. The finding of sustained thrombus formation after storage was in agreement with those of in vitro experiments using Impact-R and aorta segments. However, the thrombus formation ability was the highest immediately after the PRT treatment in the collagen column method, which was clearly different from those in the other two experiments. Using Impact-R, the surface coverage by aggregates of the PRT-treated PLTs on the day after PRT treatment was higher than that of the control PLTs, but there was no significant difference in the average size of aggregates between them.[10] In the experiments using rabbit aorta segments, the adhesive and cohesive functions of the PRT-treated PLTs were higher on Day 5 but lower on the day of PRT treatment compared with those of the control PLTs.[11] Thus, the evaluation of the ability of thrombus formation of PRT-treated PLTs may differ depending on the assay system used, and this awaits careful observation in clinical studies.

We considered that αIIbβ3 is involved in the enhancement of thrombus formation in the PRT-treated PLTs. The Mirasol PRT system uses UV radiation in a wide range of wavelengths from UV-A to UV-C (265 to 370 nm).[12,21] Verhaar and colleagues[22] reported that UV-C (254 nm) breaks disulfide bonds of αIIbβ3 and causes conformational changes of αIIbβ3, which leads to the enhancement of fibrinogen binding. Zhi and coworkers[23] suggested that UV-B (290 to 320 nm) also activates αIIbβ3 and enhances fibrinogen binding and that protein kinase C is involved in αIIbβ3 activation. Whereas they examined the effects of UV radiation alone, we showed in this study that the Mirasol PRT system, which uses a photosensitizer as well, also induced the marked activation of αIIbβ3 and enhanced fibrinogen binding. Interestingly, αIIbβ3 activated by PRT treatment did not bind fibrinogen completely immediately after the treatment. Although the level of αIIbβ3 activation induced by PRT treatment decreased with storage time, the level of fibrinogen binding on PLTs slightly increased, indicating that the activation state of αIIbβ3 represented as PAC-1 binding did not correlate with fibrinogen binding.

We speculate that, although slight conformational changes recognized by the PAC-1 antibody occurred, αIIbβ3 activation induced by PRT treatment did not cause sufficient conformational changes for αIIbβ3 to bind fibrinogen, which presents a distinct status from αIIbβ3 activation induced by physiologic agonists such as thrombin.[24] Takagi and colleagues[25] reported that integrins exist in at least three conformational states depending on the activation level: a bent conformer, an extended conformer with a closed headpiece, and an extended conformer with an open headpiece. Bunch[26] suggested that the conformational change of αIIbβ3 needs an increase in both affinity and avidity to strongly bind fibrinogen. It is thus possible that αIIbβ3 activation induced by PRT treatment is only partial so that a proportion of αIIbβ3 molecules on the PLT surface may bind fibrinogen or those molecules may weakly bind fibrinogen. The reason why the level of fibrinogen binding onto αIIbβ3 increased during storage was unclear. There may be effects of intrinsic activation of αIIbβ3 associated with the PLT storage lesion.

We found a strong correlation between thrombus formation assayed by the collagen column method and αIIbβ3 activation represented as PAC-1 binding. There was no correlation between thrombus formation and total αIIbβ3 level represented as CD41 positivity or fibrinogen binding. It is thus possible that the conformational changes of αIIbβ3 caused by PRT treatment enhanced the thrombus formation on collagen-coated beads. Jackson[27] reported that the contact of PLTs with collagen or the von Willebrand factor under various flow conditions activates αIIbβ3 and promotes thrombus growth. It seems that αIIbβ3 rapidly shifts from being partially activated to fully activated when the PRT-treated PLTs come into contact under flow with the collagen on the beads, leading to the enhanced thrombus formation. The thrombus formation assay by the collagen column method seemed to reflect the activation state of αIIbβ3 in PRT-treated PLTs quite well.

Both the thrombus formation and the PAC-1 binding to αIIbβ3 on the PRT-treated PLTs were reduced on Day 5. When Mn^{2+} was added to the PLTs, the αIIbβ3 on the PRT-treated PLTs as well as that in the control PLTs was activated, resulting in the restoration of thrombus formation. Mn^{2+} directly and fully activates αIIbβ3 without being mediated by inside-out signals from within the PLTs and enables the binding of αIIbβ3 to fibrinogen and

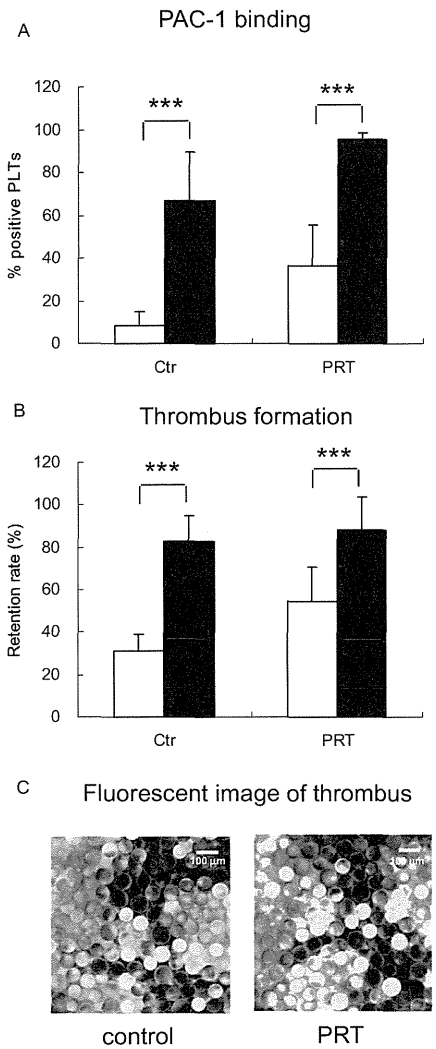

Fig. 4. Effects of Mn^{2+} on αIIbβ3 activation and thrombus formation. (A) Activated αIIbβ3 represented as PAC-1 binding on Day 5 control PLTs (Ctr, n = 7) and Day 5 PRT-treated PLTs (PRT, n = 24) in the presence (■) or absence (□) of Mn^{2+}. (B) Enhancement of thrombus formation determined by collagen column method in Day 5 control PLTs (Ctr, n = 7) and Day 5 PRT-treated PLTs (PRT, n = 24) in the presence (■) or absence (□) of Mn^{2+}. (C) Typical fluorescent image of thrombus on collagen-coated beads through which Day 5 PLTs incubated with Mn^{2+} passed: (left) control, (right) PRT-treated. Thrombus formation was determined on the basis of retention rate (%) presented as mean ± SD. ***p < 0.001.

fibrin.[19,28,29] The result showed a sufficient potential of reactivation of Day 5 PRT-treated PLTs in terms of αIIbβ3 activation and the restoration of thrombus formation in the column, suggesting that the functions of αIIbβ3 are not impaired. After immediate activation by PRT treatment, the αIIbβ3 in the PRT-treated PLTs gradually returned to the status with lower-level activation during storage. However, it was intriguing that the activation state lasted as long as 5 days with decreasing trend that seemed reversible.[30,31] Although we could not find an explanation for this reversible and long-lasting αIIbβ3 activation, it may be related to the activation state of αIIbβ3 that did not bind fibrinogen.[26,32]

In this study, thrombus formation ability was measured by the collagen column method as an index of PLT function. In the collagen column method, however, only the reaction between PLTs and collagen, the major component of the subendothelium, was evaluated in the absence of RBCs. The reaction between PLTs and collagen is important for evaluating the PLT function in the initial phase of thrombus formation. However, how much this reaction contributes to the complicated in vivo thrombus formation process has not been fully clarified yet. In addition, how the insufficient αIIbβ3 activation in PRT-treated PLTs leads to the enhancement of thrombus formation on collagen is unclear in our study. However, it has been reported that the signaling from the collagen receptor regulates αIIbβ3 activation and the subsequent thrombus formation.[33] We consider that there is a close relationship between the enhancement of thrombus formation and insufficient αIIbβ3 activation.

Although our results show the increased and sustained capacity of binding of PRT-treated PLTs to collagen, they indicate a possibility of unwanted binding of treated PLTs to undamaged endothelial cells under physiologic conditions. Although we do not have any expectation on this issue with our current system, we are currently developing an aggregometric system that could evaluate PLT aggregation caused by shear stress in the absence of collagen.

In conclusion, our studies using the collagen column method indicated that PRT treatment enhanced the thrombus formation on collagen and that the ability of the thrombus formation was well maintained during the storage period. We consider that the sustained αIIbβ3 activation is related to the enhancement of thrombus formation. In the absence of the contact of PLTs with collagen, however, αIIbβ3 activation induced by PRT treatment may be only partial. Upon contact with collagen, thrombus is formed completely along with the reactivation of αIIbβ3. These results could have an implication in the clinical setting of PLT transfusion in that the modifications of surface glycoproteins on PLTs caused by PRT treatment might not significantly affect the hemostatic effects of PRT-treated PLTs when transfused. Ultimately, the clinical

impacts of these findings on the hemostatic or even thrombogenic potential of PRT-treated PLTs should be evaluated in appropriate clinical trials.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The disposables and the instrumentation for conducting pathogen reduction by the Mirasol system were provided without charge by Terumo BCT. Otherwise, none of the authors of this article have any conflicts of interest to report as defined by AABB's policy.

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Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors

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BACKGROUND: The current prevalence of cytomegalovirus (CMV) in Japan and the risk of CMV transfusion transmission are unknown in the era of seronegative leukoreduced blood components.

STUDY DESIGN AND METHODS: We measured CMV-specific immunoglobulin (Ig)M and IgG in 2400 samples of whole blood collected from 12 groups of blood donors categorized by sex and age at 10-year intervals from their teens to their 60s. We also tested for CMV DNA using polymerase chain reaction in the cellular fractions of all samples.

RESULTS: We found that 76.6% of blood donors were CMV seropositive. The seroprevalences among donors in their 20s and 30s were 58.3 and 73.3%, respectively. We detected CMV DNA in the cellular fraction of 4.3% of samples from donors in their 60s and in 1.0% of samples from donors younger than 60 years. None of the 562 seronegative samples was DNA positive. Furthermore, 14% of DNA-positive samples also contained DNA in the plasma fraction, and two of five such samples were derived from donors in their 60s. Leukoreduced plasma components derived from donations with CMV DNA in plasma samples also contained a relevant amount of CMV DNA.

CONCLUSION: The seroprevalence of CMV among Japanese blood donors of child-bearing age has not changed over the past 15 years. Latent CMV becomes reactivated more frequently among elderly donors than among younger donors. A proportion of them have free CMV DNA in their plasma fraction, which could not be diminished by leukoreduction. The risk of transfusion-transmitted CMV infection in blood with plasma CMV DNA should be determined.

Human cytomegalovirus (CMV; *Human herpesvirus 5*) ubiquitously infects humans and persists in a latent form for long periods. It can cause asymptomatic infection in the general population or a mononucleosis-like syndrome or transient hepatitis in some healthy individuals. However, it can cause serious morbidity and mortality in immunocompromised hosts, and congenital or perinatal CMV infection causes developmental abnormalities in newborns. Morbidity can arise due to either primary infection or CMV reactivation. The transfusion of blood contaminated with CMV could be a source of primary infection in seronegative patients. Thus, CMV-safe blood components are typically required for transfusing seronegative patients who will undergo marrow or organ transplantation, patients with immunodeficiency syndrome, or premature infants. Blood facilities have implemented serologic screening of donated blood for CMV-specific immunoglobulin (Ig)G to mitigate the incidence of transfusion-transmitted CMV infection (TT-CMV) in such patients. This is conducted universally or in response to requests from physicians and has largely prevented TT-CMV infection.[1]

Leukoreduction using white blood cell (WBC) filters has been widely implemented in blood facilities to help reduce the side effects of residual WBCs in blood components such as febrile reactions or alloimmunization against WBC antigen. Leukoreduction under good

ABBREVIATION: TT-CMV = transfusion-transmitted cytomegalovirus infection.

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manufacturing practices could also abrogate the transmission of WBC-associated virus such as CMV, Epstein-Barr virus, or human T cell leukemia virus. Thus, leukoreduced blood components have been advocated as an alternative to transfusion for patients at risk for CMV when seronegative blood is unavailable, although whether leukoreduced blood is as safe as seronegative blood in terms of TT-CMV risk remains a matter of debate.[2-5]

Breakthrough cases have been attributed to transfusion with CMV-seronegative, but CMV DNA–positive blood that might have been donated during a window period, namely, the preseroconversion viremic phase of acute infection.[6] This could justify using leukoreduced blood to avoid transfusion with blood obtained during window periods that serologic screening could miss.[7] Thus, seronegative leukoreduced blood components are currently regarded as the safest strategy to prevent TT-CMV. However, Ziemann and colleagues[8] recently reported that up to 2.9% of plasma derived from donors during the window period contains CMV DNA. Because leukofiltration might not efficiently remove free CMV from the plasma fraction, this would pose another TT-CMV risk that could not be overcome by combining the two strategies.

We screened blood samples (n = 2400) donated equally by male and female volunteers of all age categories using serologic assays and nucleic acid amplification testing (NAT) to assess the risk of CMV transmission in Japan, particularly through transfusion with leukoreduced and seronegative blood components. We established a national prevalence and demographic trend for CMV infection over a range of donor ages and found no blood samples that were both viremic and seronegative. We also found that the frequency of CMV DNA positivity was higher in samples from elderly than from younger donors.

MATERIALS AND METHODS

Blood samples

We sequentially selected whole blood samples at the Japanese Red Cross Tokyo Blood Center in November 2010, where whole blood and blood samples were collected from five prefectures around the greater Tokyo metropolitan area. The samples were allocated to 12 groups according to donor sex and age at 10-year intervals from the 20s to the 60s and from age 16 to 19 years. Each of the 12 categories comprised 200 blood samples. Whole blood collected into tubes containing ethylenediaminetetraacetic acid was separated by centrifugation, during which the separation media rose to the interface between the plasma and the cellular fraction and formed a hard gel. We could thus keep them frozen until use without the two fractions becoming mixed. The plasma fraction was analyzed by CMV serology and CMV NAT. After removing the remaining plasma and interface gel, the top portion of the cellular fraction was suspended in the same volume of phosphate-buffered saline for DNA extraction.

CMV serology assays

We tested CMV-specific IgG and IgM antibodies using automated microparticle enzyme immunoassays (EIAs) and an immunochemical automated analyzer (AxSYM CMV-G and CMV-M, Abbott Laboratories, Abbott Japan, Tokyo, Japan).

DNA extraction

We extracted DNA from the cellular fraction of blood samples using the automated DNA purification kits (QIAsymphony SP and QIAsymphony DNA Midi kits, Qiagen, Tokyo, Japan) according to the protocol provided by the manufacturer (DNA Blood 1000). The input and output sample volumes were 1200 and 200 μL, respectively. Plasma DNA was likewise extracted from samples that were positive for DNA in the cellular fraction using a virus and bacteria detection kit (QIAsymphony Midi kit, Qiagen) with its accompanying protocol (Virus Cellfree 1000). The input and output sample volumes were 1.0 mL and 60 μL, respectively.

CMV NAT

We detected CMV DNA using TaqMan PCR and an sequence detection system (ABI PRISM7900HT, Applied Biosystems, Tokyo, Japan) and artus CMV TM PCR kits (Qiagen) according to the manufacturer's instructions.

We also prepared an in-house TaqMan PCR to detect CMV DNA. This system amplifies a 58-bp fragment of the UL83 gene that encodes phosphorylated 65-kDa proteins (pp65). The forward and reverse primers were 5'-TGCC ATACGCCTTCCAATTC-3' and 5'-TGGCTACGGTTCAG GGTCA-3', respectively. The TaqMan probe, 5'-CGGT AGATGTCGTTGGC-3', was labeled with a reporter dye (6-carboxyfluorescein, FAM) at the 5' end and a minor groove binder at the 3' end. The amplification reagent was supplied with a probe PCR kit (QuantiTect, Qiagen). Each reaction mixture comprised 30 μL of master mix and 20 μL of extracted DNA (equivalent to 120 μL of original sample). The thermocycling protocol comprised 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The nucleic acid concentration was calculated by measuring the absorbance of the extracted DNA at 260 nm.

A validation study for PCR sensitivity included NATtrol NATCMV-0004 (ZeptoMetrix, Buffalo, NY) as the external reference CMV for both PCR analyses. The reference solution was serially diluted in 5% bovine serum albumin (BSA) and portioned into small tubes for PCR analysis over a period of 4 days. We tested CMV concentrations five times daily for each PCR procedure, for a total

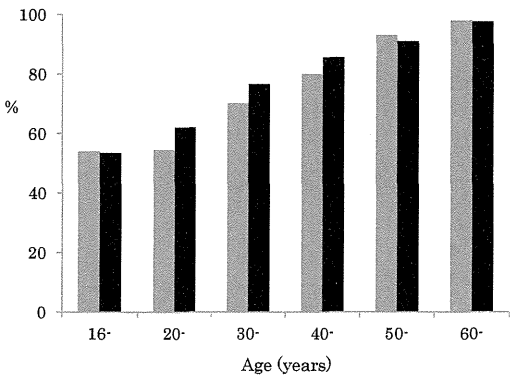

Fig. 1. Age distribution of CMV-specific IgG prevalences in (▒) men and (■) women.

of 20 replicates at each concentration. We then calculated the 95 and 50% limits of detection for each PCR using probit analysis. Correlation study between the reference solution and first World Health Organization international standard (NIBSC 09/162) revealed that 32.3 genome equivalents/mL (geq/mL) was equivalent to 1 IU/mL. Samples in which the PCR results were ambiguous were further analyzed using nested PCR targeting the UL139 sequence as described by Bradley and colleagues[9] with the modification for DNA polymerase (KAPA DNA polymerase, Nippon Genetics, Tokyo, Japan).

To adjust the amount of CMV DNA for the number of WBCs in the sample, we estimated the number of Exon 5 sequences of CD81 in specimens using real-time PCR.[10] CD81 was chosen as a marker of WBCs as it is present with two haploids in a cell. Amounts of CMV DNA are described as geq per 6.0×10^6 WBCs (geq/PBL unit) in this study. The lowest limit of quantitative CMV DNA detection was 40 geq/mL before adjustment for WBC numbers.

Statistical analysis

Data were analyzed using computer software (SSRI, Excel Statistics, Version 8, Social Survey Research Information, Tokyo, Japan; for Windows, Microsoft Excel 2007, Tokyo, Japan). Significance was determined using the chi-square test and t test. p values of less than 0.05 were considered significant.

RESULTS

We initially examined the prevalence of anti-CMV among Japanese blood donors. Figure 1 shows the prevalence of specific IgG among the age categories. The prevalence exceeded 50% even in male and female teenagers and steadily increased over time to reach nearly 100% in their 60s. Although not significant, the prevalence tended to be higher in females than in males aged from the 20s to the 40s. The increase in the prevalence was the highest between the 20s and 30s (15%; combined for both sexes) and gradually decreased with age to 5.8% between the 50s and 60s. The mean prevalence in the six age categories was 76.3%. The overall CMV prevalence adjusted for an assumed population with the age distribution of Japanese blood donors (Japanese Red Cross data, 2010) was 76.6%. The IgM prevalence was higher among females than males between the ages of 16 and 39 years ($p < 0.05$, Table 1). Seven donors were IgM positive and IgG negative, and four of them were teenagers.

We next examined the presence of CMV DNA in the cellular fraction of 2400 whole blood samples. A validation study showed that the 95 and 50% limits of CMV DNA detection for artus CMV TM PCR were 41.6 and 5.3 geq/mL, respectively, and those for the in-house PCR were 29.6 and 5.4 geq/mL, respectively (Table 2). Only samples that were positive for at least two PCR analyses including nested PCR targeting the UL139 sequence were defined as CMV DNA positive. We identified 37 samples that were positive for CMV DNA in the cellular fraction (Table 3). Four other samples were positive for only one PCR analysis and were defined as DNA indeterminate. Table 4 shows the relationship between DNA positivity and the serostatus of the specific antibody. We found DNA positivity in six (6.6%) of 91 samples that were both IgM and IgG positive and in 31 (1.8%) of 1740 that were only IgG positive. Although the samples that were positive only for IgM did not contain any that were DNA positive, the frequency of DNA positivity was significantly higher in six (6.12%) of 98 samples that were IgM positive with or without IgG than in those that were positive only for IgG ($p < 0.03$). Viral load was significantly higher in CMV DNA–positive samples that were both IgM and IgG positive (mean, 670 geq/PBL unit) than in those that were only IgG positive (170 geq/PBL unit, $p < 0.03$, t test). Notably, none of the 562 samples that were both IgM and IgG negative was also DNA positive.

Table 5 compares the distribution of 37 DNA-positive samples with age categories. The frequency of DNA positivity was significantly higher (17/400, 4.3%) among donors in their 60s than in any other age category (0.8%-1.3%, $p < 0.03$) from the teens to the 50s or the combined age category (1.0%, $p < 0.03$) from 16 to 59 years. The range of viral load in the 37 DNA-positive samples was between less than 40 and 3.4×10^3 geq/PBL unit (mean, 250 geq/PBL unit; median, 80 geq/PBL unit). The difference in viral load in the samples between donors aged less than 60 years (mean, 310 geq/PBL unit) and those in their 60s (mean, 170 geq/PBL unit) was not significant. The presence of DNA in the plasma fraction was further investigated in these 37 samples. Five (13.5%) of them were plasma DNA positive with a viral load between less than

TABLE 1. Prevalence of CMV-specific IgM among blood donors*

	Male		Female		Total	
Age (years)	Positivity	Percent	Positivity	Percent	Positivity	Percent
16-19	6	3.0†	13 (4)	6.5†	19 (4)	4.8
20-29	5 (1)	2.5†	15	7.5†	20 (1)	5.0
30-39	5 (1)	2.5†	13	6.5†	18 (1)	4.5
40-49	8	4.0	10	5.0	18	4.5
50-59	5 (1)	2.5	3	1.5	8 (1)	2.0
60-69	6	3.0	9	4.5	15	3.8
Total	35 (3)	2.9	63 (4)	5.3	98 (7)	4.1

* Numbers of donors positive only for specific IgM are shown in parentheses.
† IgM prevalence significantly higher among female donors than among male donors (16-19, 20-29, and 30-39 years); chi-square test ($p < 0.05$).

40 and 5.8×10^3 geq/mL (median, 170 geq/mL). These five were scattered across age categories with two being in their 60s. One sample obtained from a teenaged donor was IgM and IgG positive and the other four were positive only for IgG. We identified two more samples from donors in their 60s that were DNA positive with only one PCR analysis.

We interdicted three components of fresh-frozen plasma that had CMV DNA in the plasma fraction. All of them were derived from whole blood that was leukoreduced before storage. We also detected CMV DNA in all three plasma components. One component donated by the IgM- and IgG-positive teenaged male donor contained 9.7×10^3 geq/mL CMV DNA and the other two components that were positive only for IgG from donors in their 60s contained 1.9×10^2 and 1.6×10^3 geq/mL CMV DNA.

DISCUSSION

We investigated the prevalence of CMV among Japanese blood donors categorized by sex and age at 10-year intervals. The more than 50% prevalence of CMV infection among individuals aged between 16 and 19 years is in contrast with the approximately 30%[11] prevalence in other developed countries. The increase in the prevalence (15%) between donors aged in their 20s and 30s implies that young adults become infected with CMV at a rate of 1.5% per annum. This is similar to the annual rate of 1.69% observed between 1994 and 1999,[12] implying that the risk of CMV infection among females of child-bearing age that is directly related to symptomatic fetal CMV infection has not changed over the past 15 years. The reason for the sustained high prevalence in Japan is unclear, but prolonged breast-feeding and communal child care practices in Japan probably influenced the rates in younger donors. The prevalence in Japan has become almost maximal after the age of 60 years, which contrasts with the continuous lifelong primary infection found in other developed countries.[11] The CMV seroconversion rate (1.33%)[11] among German blood donors aged 30 to 35 years is close to the 1.5% rate of increase described above. However, care must be taken in comparing the present results with those of the German study because our results were generated from a cross-sectional study whereas the German findings were obtained through longitudinal follow-up of seronegative donors. Although insignificant, the prevalence in females tended to increase sooner than in males, a finding that is consistent with the higher prevalence of specific IgM in younger females than in younger males.[13,14]

We detected CMV DNA in the cellular fraction of 1.7% (41/2400) of all, or 2.2% (41/1831) of the seropositive, samples with or without specific IgM. This frequency was comparable to those reported by Greenlee and colleagues[15] and Roback and colleagues.[16] We found CMV DNA more frequently in samples that were IgM positive than in those that were only IgG positive (6.12% vs. 2.0%, $p < 0.03$), indicating that active CMV replication occurs more frequently during acute primary infection that is often accompanied by IgM positivity. None of the samples from the group of seven donors that was positive only for IgM was CMV DNA positive. This is reasonable because Ziemann and coworkers[17] detected CMV DNA only in 10% of 148 primary seroconverted blood donors. At that rate we would be unable to identify a single DNA-positive individual in our study population. The same authors showed that CMV DNA levels peak during the late phase of primary infection in newly seropositive donors.[8] Although whether a rationale exists for introducing screening for specific IgM in addition to IgG remains to be determined,[18] the chemiluminescence tests for CMV currently applied by the Japanese Red Cross detect only IgG. Although we have discussed seroprevalence and its relationship with the presence of DNA by interpreting IgM positivity as representing primary infection, reactivity for CMV-specific IgM measured by EIAs must be considered with caution. Several articles have reported frequent nonspecific reactions[19,20] and suggest including Western blot analyses or IgG avidity assays to ensure reactivity. Because of the small plasma volume of most of the donor samples, we were unable to apply these analyses. Thus, the above findings and our interpretations based on categories by IgM positivity might be inconclusive and require further investigation.

We found no DNA-positive samples among 562 that were seronegative, suggesting that the likelihood of donating DNA-positive blood during the window period[7] is very low in Japan. This finding is similar to that described by Roback and coworkers,[16] who found no CMV DNA positivity among 514 healthy, seronegative blood donors. However, these findings do not allow underestimation of

TABLE 2. Determination of sensitivity of two PCR systems by replicate testing and probit analysis

PCR	Ref* (geq/mL)	D 1	D 2	D 3	D 4	Total	95% LOD (geq/mL)	50% LOD (geq/mL)
Artus CMV	1	1/5	1/5	1/5	1/5	4/20	41.6	5.3
	5	5/5	3/5	2/5	5/5	15/20		
	10	5/5	5/5	4/5	4/5	18/20		
	50	5/5	5/5	5/5	5/5	20/20		
In house (UL83)	1	0/5	1/5	0/5	1/5	2/20	29.6	5.4
	5	5/5	3/5	5/5	4/5	17/20		
	10	5/5	5/5	4/5	5/5	19/20		
	50	5/5	5/5	5/5	5/5	20/20		

* NATtrol as CMV reference was diluted in 5% BSA.
artus = artus CMV TM PCR kits (Qiagen); D = day; in house (UL83) = in-house PCR targeting CMV UL83 sequence; LOD = limit of detection.

TABLE 3. CMV DNA–positive samples and PCR procedures

PCR results	Number of samples
UL83 positive and artus positive	29
UL83 repeatedly positive	2
artus repeatedly positive	2
UL83 positive and UL139 positive	3
artus positive and UL139 positive	1
Indeterminate*	4
Negative	2359
Total	2400

* Positive in only one PCR analysis.
artus = artus CMV TM PCR kits (Qiagen); UL139 = PCR targeting CMV UL139 sequence; UL83 = in-house PCR targeting CMV UL83 sequence.

the risk of TT-CMV caused by transfusion with window period–derived blood components because we did not focus on blood samples obtained at the time of acute primary infection when CMV replication is most likely to be active. In fact, Ziemann and colleagues[8] found that two (2.9%) samples were DNA positive among 68 plasma samples obtained from final seronegative donations during the course of seroconversion. Collectively, a risk of TT-CMV related to window period donation exists but the frequency seems very low.

The frequency of detecting CMV DNA was 4.3% among donors in their 60s, compared with 1.0% (0.8%-1.3%) in the population aged from 16 to 59 years. Considering that the specific IgG prevalence has already peaked by age 60 years in Japan, the notion that the DNA-positive individuals in their 60s were nonimmune to CMV and emitted CMV virions during the course of primary CMV infection is inconceivable. Latent CMV more likely became reactivated in those elderly individuals. The reactivation of CMV in elderly persons is thought to represent "immunosenescence" caused by chronic CMV infection.[21,22] The current concept of immunosenescence in relation to CMV infection is that terminally differentiated memory T cells accumulate with ageing in the limited peripheral "immunologic space," which causes a progressive decline in the generation of naive T cells that protect against new pathogens. In addition, a considerable portion of the accumulated memory T cells were specific for CMV.[23-25] Thus, CMV infection is considered a driving force or risk biomarker for the constitution of a skewed peripheral T-cell repertoire. Despite conflicting results and ideas about epidemiology and immunologic mechanisms, the clinical impact of the CMV infection on individuals who are not immunocompromised has remained a central question.[22] Whether or not all persons with CMV infection acquire skewed T-cell phenotypes with aging, the kinds of socioeconomic or physical factors that facilitate this process, and when this process starts to compromise the immune system should be addressed. We established statistical evidence of CMV reactivation occurring in the peripheral blood of voluntary blood donors in their 60s. Viral load did not significantly differ between donors in their 60s and those aged less than 60 years. Blood donors in Japan are supposedly healthy individuals who have all been qualified by questionnaires and consultation with physicians. Our results therefore suggest that CMV reactivation is a constitutional event in CMV carriers and starts to occur during the sixth decade of life, although the possibility remains that donors positive for CMV DNA recently might have had specific illnesses or behaviors that are related to CMV reactivation. The findings of animal experiments suggest that lytic viral reactivation is necessary to establish the peripheral T-cell repertoire skewed for CMV.[26] Stowe and colleagues[27] detected CMV in 57% of urine samples from elderly individuals (66 to 83 years) but in none of those from younger individuals (25 to 55 years). This would also suggest that CMV reactivation occurs more frequently among elderly, than younger, individuals, although they did not detect CMV in any blood samples from both groups. However, this might have resulted from the small sample size studied (11 elderly individuals compared with 400 aged ≥60 years in this study). The rather clear cutoff of the reactivation frequency between the 50s and 60s is reminiscent of a Swedish study[28] showing increased 10-year mortality

TABLE 4. Association between CMV serostatus and CMV DNA positivity

Serology status	Number of samples	DNA+ (n)	Ratio (%)	Viral load Mean/median Range (geq/PBL unit)
IgM–/IgG–	562	0	0	
IgM+/IgG–	7	0	0*	
IgM+/IgG+	91	6	6.6*	670/62† <40-3400
IgM–/IgG+	1740	31	1.8*	170/80† <40-920
Total	2400	37	1.5	

* DNA positivity ratio significantly higher in IgM+ than in IgM–/IgG+ samples (6 [6.1%] of 98 vs. 31 [1.8%] of 1740); chi-square test ($p < 0.03$).
† Viral load in IgM+/IgG+ samples significantly higher than that in IgM–/IgG+ samples; t test ($p < 0.03$).
DNA+ = DNA positive.

TABLE 5. Age distribution of CMV DNA positivity in cellular and plasma fractions*

Age (years)	Cellular fraction DNA+	Cellular fraction Ratio (%)	Plasma fraction, DNA+
16-19	4 (2)	1.0	1 (1)
20-29	5	1.3	1
30-39	5 (1)	1.3	0
40-49	3 (2)	0.8	0
50-59	3	0.8	1
16-59 total	20 (5)	1.0	3 (1)
60-69	17 (1)	4.3	2†
Total	37 (6)	1.5	5

* Numbers of donors specifically positive for both IgM and IgG are shown in parentheses.
† In addition to these two samples, two others from donors in their 60s were positive for one PCR analysis.
DNA+ = DNA positive.

rates among individuals with immune risk profiles at the age of 65 years but not at the age of 55 years.

We identified five (13.5%) samples that were positive for CMV DNA in the plasma fraction of 37 blood samples that contained CMV DNA in the cellular fraction. This result is comparable to the report by Ziemann and coworkers[8] in which 44% of blood samples from 82 recently seroconverted donors were CMV DNA positive in the plasma fraction. Drew and colleagues[6] also reported that three of 384 samples obtained from 192 seroconverted blood donors contained low plasma levels of CMV DNA. We quantified CMV DNA levels in three plasma products derived from donations that had CMV DNA in plasma samples. The DNA levels (1.9×10^2 to 9.7×10^3 geq/mL) were comparable to those measured in the plasma samples. Because all blood components including apheresis-derived plasma components are leukoreduced in Japan, this finding indicated that leukofiltration cannot reduce levels of free CMV DNA in the plasma fraction.

The identification of blood donations with plasma fractions containing CMV DNA raises concerns about the safety of blood components. The residual risk of TT-CMV under the current blood program that applies both seroscreening and universal leukoreduction could be focused on blood with plasma viremia that is provided during the window period because plasma viremia might not be appropriately managed by leukofiltration. This leads to the notion of Ziemann and colleagues[8] that leukoreduced components would be safer when obtained from seropositive donors at least 1 year after seroconversion. This is also based on the finding that plasma viremia has barely been detectable among donors who remain seropositive for more than 1 year. However, our study showed that a proportion of latently infected individuals presents with free CMV DNA in plasma fractions. Free CMV DNA in plasma could not be effectively diminished by prestorage leukoreduction, which was verified by assays of leukoreduced plasma products. Therefore, the strategy suggested by Ziemann and colleagues, while eliminating window period–related risk, might generate another risk associated with blood containing free plasma CMV DNA that is provided mainly by elderly donors. Although we identified only two samples from donors in their 60s that were plasma DNA positive, one of them related to a plasma product containing 1.6×10^3 geq/mL CMV DNA, a viral load that was comparable to that for window period donation provided by the teenager (9.7×10^3 geq/mL). Moreover, we found two other samples with possible plasma DNA among donors in their 60s, although they were DNA positive only for a single PCR analysis, suggesting low DNA concentrations. Whereas we found that whole blood CMV DNA positivity among donors in their 60s was 4.3%, that identified in an elderly US population with a mean age of 84.5 years was 42.3%.[29] Thus, since the frequency of whole blood CMV increases dramatically after 60 years, we can speculate that the frequency of plasma CMV also increases with age. In this context, serious problems could arise in countries that accept donors over 70 years of age if seropositive donations are accepted for transfusion into patients at risk. Although leukoreduced blood components have been advocated as an alternative when seronegative blood is not available, they might carry a higher risk of TT-CMV

than seronegative blood, which might partly explain higher TT-CMV frequency among patients transfused with leukoreduced blood compared with seronegative blood.[2,4]

Further study is required to compare possible TT-CMV risks between persistently seropositive blood that might contain free CMV virions due to reactivation and seronegative blood that might incidentally contain such virions due to window period donation. Ziemann and coworkers concluded from a recent extensive study of more than 22,000 samples that TT-CMV risk is essentially comparable between window period donations among seronegative donors and donations with reactivation among long-term seropositive donors.[30] Before assessing the TT-CMV risk in Japan, the degree to which window period donation constitutes the blood donor population will need to be determined. Other basic issues also need to be resolved. Because we used techniques involving DNA amplification but not viral culture for plasma study, whether CMV DNA identified in plasma with this technique constitutes replication-competent virions remains unknown.[31,32] Whether plasma products containing free CMV virions is infectious through blood transfusion also needs to be resolved.[13,33] Whether blood components containing CMV virions possibly derived from reactivation in latently infected blood donors are as infectious as those derived from donations provided during acute primary infection also requires investigation. The clinical relevance of CMV neutralizing antibody that can be found in latently infected individuals also must be considered. Finally, the minimal infectious dose of CMV virions acquired through blood transfusion should be determined for each type of blood component.

In conclusion, the seroprevalence of CMV among a Japanese population of blood donors was 76.6%. The prevalence among donors in their 20s and 30s has not changed over the past 15 years. We detected CMV DNA in 1.7% of 2400 samples. None of the 562 seronegative samples was DNA positive. We detected CMV DNA more frequently in blood from donors aged in their 60s than from younger donors. Among DNA-positive samples, 14% contained DNA in the plasma fraction, and this frequency might be higher among donors in their 60s than younger donors. CMV DNA persists in a portion of seropositive blood even after prestorage leukoreduction and leukoreduced blood without seroscreening might not be as safe as seronegative blood in terms of TT-CMV risk. The risk of TT-CMV in blood with detectable CMV DNA in the plasma fraction should be determined.

CONFLICT OF INTEREST

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Interstitial fluid shifts to plasma compartment during blood donation

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BACKGROUND: A vasovagal reaction (VVR) occurs in 0.8% to 0.9% of voluntary blood donors in Japan. However, they generally tolerate the acute loss of 400 mL of whole blood rather well, perhaps because several circulatory defense mechanisms compensate for the loss. This study aimed to determine the extent to which an interstitial fluid shift contributes to the development of a VVR.

STUDY DESIGN AND METHODS: Blood hemoglobin (Hb) was measured upon admission, at venipuncture, and immediately after collecting 400 mL of whole blood from 736 donors. Shifted fluid volume was calculated using a formula that included Hb levels and estimated total blood volume.

RESULTS: By the end of blood collection, 188 ± 80 and 211 ± 82 mL of fluid, which is equivalent to almost half of the total amount of withdrawn blood, had entered the intravascular space in male and female donors, respectively. The difference between the sexes was significant despite the lower body weight and circulating blood volume of the female donors. Body weight increased, whereas age decreased the volume of shifted fluid in female donors.

CONCLUSION: Blood loss after donation is quickly compensated by an interstitial fluid shift into the intravascular space and may not be the only direct cause of VVR in the setting of a whole blood donation of 400 mL.

Adverse reactions that occur among blood donors during or shortly after blood donation seriously impair the motivation of repeat donors. A vasovagal reaction (VVR) indicated by light-headedness, dizziness, and weakness is the most frequent reaction, occurring in 0.8% to 0.9% of blood donors in Japan (Japanese Red Cross Blood Centers data). Although a VVR in most individuals has a relatively moderate clinical course and spontaneously resolves, a small proportion (0.015%) of donors might faint, fall, and possibly sustain serious injuries after a donation. From this perspective, investigation into the mechanisms of VVR and the development of preventive or relief measures has been crucial for blood collection institutions.[1-4]

During the presymptomatic phase of VVR, donors have a hyperdynamic circulatory status with moderately elevated blood pressure and increased cardiac output. The symptoms of VVR coincide with a sudden decrease in arterial blood pressure and heart rate.[4-6] Although a reflex activation and/or depression of the autonomous nervous system has been implicated in the pathophysiology of the abrupt transition from hyper- to hypodynamic status,[7,8] the time course of symptoms can be explained from the viewpoint of venous return. Excessive blood pooling in capacitance vessels results in decreased venous return, followed by insufficient left ventricle filling and hence decreased cardiac output. Among the primary factors that cause blood pooling is fear of needles or pain accompanying venipuncture, which often results in the withdrawal of the sympathetic tone of blood vessels. Standing shortly

ABBREVIATION: VVR = vasovagal reaction.

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after blood donation might also contribute to blood pooling.[9-12] In the setting of blood donation in Japan, 400 mL of whole blood takes less than 10 minutes to withdraw, and this corresponds to a moderate grade of hemorrhage. Thus, routine blood donation presents a possibly problematic series of events that could facilitate insufficient venous return to the ventricles of a proportion of blood donors. A recent study has elucidated the extent to which primary vasodilation, blood volume loss, and standing posture contributes to the development of syncope.[4]

Several physiologic mechanisms could compensate for acute blood loss.[13-15] The mobilization of interstitial body fluid from organs that have a large tissue mass and fluid reservoir, such as skeletal muscle and skin, contributes to the restoration of plasma volume.[16,17] By measuring hemoglobin (Hb) concentrations before and after collecting blood from 736 blood donors, we calculated plasma volume recovery after a short period of blood collection and investigated the role of the refilling mechanism in the restoration of blood volume.

MATERIALS AND METHODS

Blood donor criteria

Individuals ranging in age from 16 to 69 years are eligible to donate blood in Japan, where 200 and 400 mL of whole blood may be collected. Males aged 17 years and all individuals aged at least 18 years are permitted to donate 400 mL of whole blood. The standard Hb concentrations in donations of 400 and 200 mL from males and females are at least 13.0 and at least 12.5 and at least 12.5 and at least 12.0 g/dL, respectively. Body weight should be at least 50 kg for 400-mL donations and at least 45 and at least 40 kg for 200-mL donations from males and females, respectively. The total volumes of whole blood withdrawn at one donation, including that for initial flow diversion, are 425 and 225 mL for 400- and 200-mL donations, respectively. Males and females may donate an annual maximum volume of 1200 and 800 mL, respectively. Only donors with systolic blood pressure of at least 90 mmHg are eligible for blood donation.

Blood donors and blood donation in this study

We analyzed data obtained only from those who donated 400 mL of whole blood between July 2008 and May 2009. All such donors who presented at the Tachikawa (a suburb of Tokyo) blood donation facility in the morning between Monday and Friday were included in this study. Changes in peripheral blood volume during the collection of 400 mL of whole blood were estimated on the basis of the Hb concentration measured at three time points during the donation process.

After an interview with a physician was completed, venous blood samples were collected from donors to determine Hb and count blood cells. Donors judged eligible for donation according to the Hb standard described above were encouraged to consume as much liquid as possible and instructed to urinate before donation. A 17-gauge intravenous catheter was placed in an antecubital vein of recumbent donors to collect whole blood. A small portion of blood for Hb measurements and blood testing and a repository sample were obtained from a diversion bag. The Hb values obtained in this manner were regarded as those at the start of blood collection. Blood collected over a period of more than 10 minutes was regarded as invalid. After the catheter was removed at the completion of blood collection, Hb was measured in 2 to 3 mL of blood that remained in the tubing of the collection bag system. This volume is usually around 7 mL and does not contain any of the anticoagulant included in the collection bag. This sample was regarded as blood in the peripheral vein at the endpoint of blood collection. Those who completed donations remained recumbent for at least 5 minutes thereafter. After it was confirmed that their blood pressure had returned to a safe range, the donors rested in another room for at least 10 minutes and consumed a beverage.

Estimation of blood volume and statistics

The baseline blood volume at admission (V_1) of each donor was calculated using Ogawa's equation[18] as $0.168H^3 + 0.050W + 0.444$ for adult males and $0.250H^3 + 0.0625W - 0.66$ for adult females, where H and W indicate donor height (m) and weight (kg), respectively. Blood volume is routinely calculated in Japan using this equation. V_1, V_2, and V_3 and Hb_1, Hb_2, and Hb_3 in the following equations represent blood volume (V) and Hb concentrations upon admission and at the start and end of blood collection, respectively. Assuming that the absolute amount of Hb remains constant and is estimated as $V \times Hb$, the relationships among them are expressed as:

$$V_1 \times Hb_1 = V_2 \times Hb_2,$$

$$V_3 \times Hb_3 = V_2 \times Hb_2 - 0.425 \times (Hb_2 + Hb_3)/2,$$

and

$$\text{shifted volume} = V_3 - (V_2 - 0.425),$$

where $0.425 \times (Hb_2 + Hb_3)/2$ represents the amount of Hb present in the donated blood. The Hb concentration was measured using an automated cell counter (Sysmex 3000, Sysmex, Tokyo, Japan) immediately after sampling at the donation site. Hb was measured daily in three samples per donor using a single-cell counter that was validated every day.

Data were statistically analyzed using software (SSRI, Excel Statistics, Version 8, Social Survey Research Informa-

TABLE 1. Profiles of blood donors enrolled in this study and volume of fluid that shifted from interstitium to plasma compartment during blood collection

Characteristic	Mean	SD	Min	Max
Males (n = 447)				
Age (years)	42.0	13.2	18	69
Height (cm)	171	6	154	189
Body weight (kg)	68.7	9.8	47.0	130.0
Blood volume (mL)	4721	534	3590	7923
Body mass index	23.5	2.9	15.9	40.1
Hb_1*	15.0	1.0	13.0	18.3
Hb_2	14.9	1.0	12.7	18.5
Hb_3	14.3	1.0	11.9	17.7
Shifted volume (mL)	188	80	25	601
V_2 (blood volume at start of blood collection): 4751 ± 547 mL				
V_3 (blood volume at the end of blood collection): 4514 ± 566 mL				
$p < 0.03$ between Hb_1 and Hb_2 and between Hb_2 and Hb_3				
Females (n = 289)				
Age (years)	39.6	14.0	18	69
Height (cm)	159	5	143	173
Body weight (kg)	57.9	6.6	47.0	82.0
Blood volume (mL)	4003	449	3062	5566
Body mass index	22.9	2.7	17.7	32.8
Hb_1	13.5	0.7	12.5	16.1
Hb_2	13.4	0.7	12.0	16.1
Hb_3	12.7	0.7	11.3	15.0
Shifted volume (mL)	211	82	31	540
V_2: 4031 ± 456; V_3: 3817 ± 478; $p < 0.03$ between Hb_1 and Hb_2 and between Hb_2 and Hb_3				

* Hb_1, Hb_2, and Hb_3: Hb concentrations upon admission and at start and end of blood collection, respectively.

tion Co. Ltd, Tokyo, Japan) for Windows (Microsoft Excel 2007, Tokyo, Japan). Statistical analysis was performed using one-sided t tests with paired or unpaired samples and chi-square test. Factors associated with shifted volumes were determined by multiple regression analysis.

RESULTS

We analyzed data derived from samples of whole blood (400 mL) donated by 736 individuals (male, n = 447; female, n = 289) during the study period. Among the male and female donors 4.7% (21/447) and 12.5% (36/289), respectively, were donating for the first time. Four male and five female donors had developed a VVR during previous blood donations. The mean interval between admission and venipuncture for blood collection averaged 19 minutes, while 93 and 90% of male and female donors, respectively, consumed 200 to 400 mL of water. We first analyzed the difference in blood volume estimated upon admission (V_1) and at the start of blood withdrawal (V_2) to determine the effect of the consumed water on the circulating blood volume. Although the Hb concentration significantly decreased from the time of admission (Hb_1) to the start of collection (Hb_2) (Table 1), the mean (±SD) increase in blood volume ($V_2 - V_1$) was only 30 ± 87 mL for males and 28 ± 88 mL for females.

The Hb concentration significantly decreased between the start and the end of collection in both sexes ($p < 0.03$, Table 1). The volumes of fluid that shifted from the interstitial space to the plasma compartment during blood collection were 188 ± 80 (mean ± SD) and 211 ± 82 mL for males and females, respectively (Table 1). The difference in the shifted volume between the two groups was significant ($p < 0.01$) and remarkable considering that the women had a lower mean body weight and lower mean circulating blood volume than men (57.9 kg vs. 68.7 kg and 4003 mL vs. 4721 mL, respectively; Table 1). Figure 1 shows a greater shifted volume in females than in males within the same range of body weight ($p < 0.01$ for all body weight groups) and that the volume increased more sharply in females than in males accompanied by increasing body weight. The difference in the shifted volume between male and female donors tended to be greater in younger donors, significant between the ages of 30 and 49 years ($p < 0.03$), and minimal at ages between 50 and 69 years (Fig. 2). The shifted volume tended to decrease with increasing age for both sexes, and the difference was significant between females in the age ranges of 30-49 (217 mL) and 50-69 (186 mL, $p < 0.01$) years. No other donor age categories were associated with a significant decrease in the shifted volume. Body mass index was not associated with the volume of shifted fluid (data not shown). Multiple regression analysis using donor age, height, and body weight as predictor variables revealed only body weight in males as an independent predictor ($p < 0.01$) of an increase in shifted volume, whereas donor age and body weight in females were independent factors for a decrease and an increase ($p < 0.01$), respectively, in shifted volume. The mean amounts of time required to collect 400 mL of blood were 8.0 and 8.8 minutes and the mean rates of fluid shift during blood collection were 0.38 ± 0.19 and 0.45 ± 0.22 mL/min/kg (mean ± SD, $p < 0.01$) for males and females, respectively. Figure 3 shows the distribution of the shifted volume by time and weight. The distribution curve for females shifted to the right of that for males (that is, greater skewness for volume).

We also investigated whether any specific features caused a lower shifted volume by assigning the donors to categories based on a shifted volume of either more or less than 150 mL (Table 2). Greater height, body weight, and blood volume, but not donor age in males, and a greater body weight, blood volume, and younger age in females were associated with a greater shift in volume (univariate

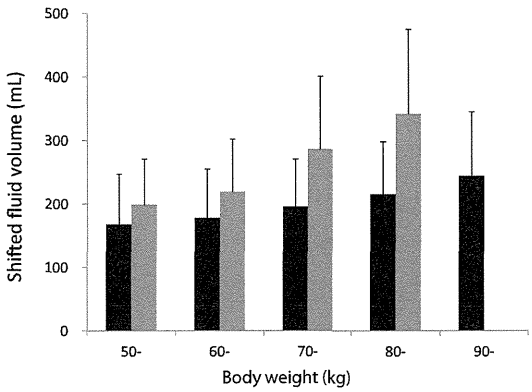

Fig. 1. Volume of shifted fluid relative to body weight of donors. (■, ▒) Mean volumes of shifted fluid in male and female donors, respectively.

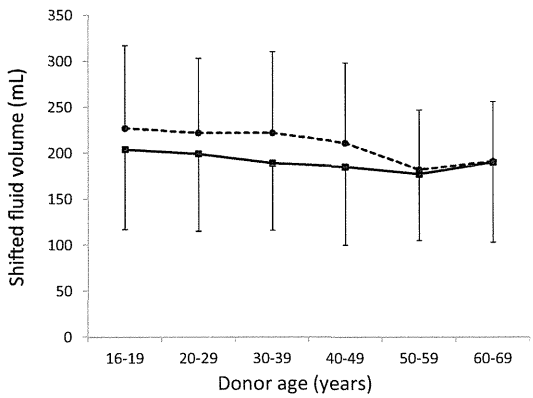

Fig. 2. Volume of shifted fluid relative to donor age. (—▪—, ---▪---) Mean volumes of shifted fluid in male and female donors, respectively.

analysis, Table 2). The proportions of male and female donors with a shifted volume of less than 100 mL was 11.0% (49/447) and 5.5% (16/289), respectively (significant difference, $p < 0.01$). The shifted fluid volume in four donors who developed relatively mild VVR symptoms shortly after donation during this study (Table 3) was relatively low at 99, 158, 163, and 197 mL.

DISCUSSION

The acute loss of 400 mL of whole blood in the blood donation setting is generally tolerated well by voluntary donors. In fact, a blood donation resulting in organ failure or systemic inflammation in Japan has never been reported. This may be because either the depletion of such moderate volume has no noticeable effect on systemic

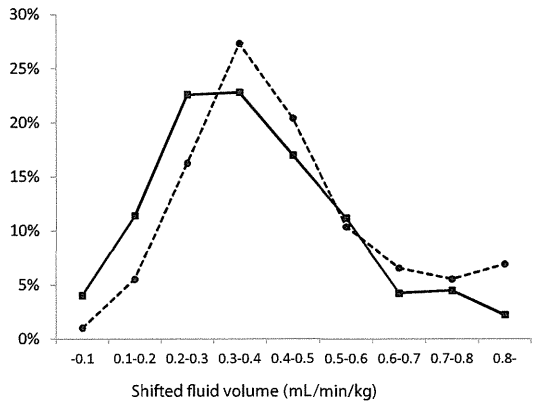

Fig. 3. Distribution of shifted fluid volume by time and weight. (—▪—, ---▪---) Ratios of male and female donors, respectively, with indicated shifted volumes.

TABLE 2. Profiles of donors with shifted fluid volumes of less than or at least 150 mL*

Characteristic	Shifted volume (mL) <150	Shifted volume (mL) ≥150	Difference
Males (n)	155	292	
Age (years)	42.8 (±13.6)	41.6 (±13.0)	>0.05
Height (cm)	169.7 (±5.7)	171.4 (±5.8)	<0.01
Body weight (kg)	66.4 (±9.0)	70.0 (±10.1)	<0.01
Blood volume (mL)	4598 (±504)	4787 (±539)	<0.01
Females (n)	68	221	
Age (years)	42.5 (±14.0)	38.8 (±14.0)	<0.05
Height (cm)	159.0 (±4.9)	159.3 (±4.9)	>0.05
Body weight (kg)	56.6 (±5.5)	58.4 (±6.8)	<0.05
Blood volume (mL)	3903 (±374)	4034 (±465)	<0.05

* Data are reported as mean (±SD).

blood circulation and/or because several circulatory defense mechanisms collaborate to restore physiologic blood distribution. Nevertheless, VVR indicated by temporary hypotension accompanied by decreased cardiac output does arise in a proportion of healthy blood donors.

Although the precise physiologic mechanism of VVR observed during the blood donation setting has not been fully elucidated, it might be initiated by the primary withdrawal of sympathetic vasoconstrictor tone in blood vessels. Although arterial blood pressure can be momentarily maintained due to the autonomous reflex mechanism, impending hypotension finally becomes uncompensated and symptomatic. The decrease in blood pressure could be a result of the combined effects of a vasodepressive reflex with or without a cardioinhibitory reflex and a failure to increase venous return. Impaired venous return would be caused by the dilation of capacitance vessels, an orthostatic effect after donation,[4,9] and decreased circulating blood volume as a result of blood withdrawal. Whole blood is collected in quantities of 200